Remarks

Claims 1-24 are pending. Allowance of claims 1-7, 9, 11 and 13-22 is greatly appreciated. Claims 19 and 20 have been amended to correct claim language.

Rejection Under 35 U.S.C. § 112, second paragraph

Claims 8, 10, 12, 23 and 24 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

The Examiner alleged that the structural formula for an oligoamine recited in claim 8 has an R inside a circle, which is unclear. In addition, the claim recites x+y+z+, which appears incomplete. Claim 8 is amended to correct typos in the language. Support for the amendments can be found in the specification on page 9, lines 17-20.

Definiteness of claim language must be analyzed, not in a vacuum, but in light of the content of the particular application disclosure, the teachings of the prior art, and the claim interpretation that would be given by one possessing the ordinary skill in the pertinent art at the time the invention was made.

Claim 10 is amended to read "a spermine", which one of ordinary skill in the art would understand to mean spermine and derivatives thereof. For example, on page 11, line 7, the specification discloses alkylated spermine. In addition, in Example 2 on page 41, line 3, the specification discloses a hydrazide derivative of spermine. Furthermore, spermine derivatives such as TM-TPS (N,N',N",N"'-

tetramethyl-N,N',N",N"'-tetrapalmityl **spermine**), DOSPA (2,3-dioleyloxy-N-[2(**sperminecarboxamido**)ethyl]-N,N-dimethyl-1-pro-pan-eammonium trifluoroacetate), and DOGS (dioctadecylamidoglycyl **spermine**) were well known in the art (see, for example, the enclosed copy of Kichler A. et al. "Influence of the DNA complexation medium on the transfection efficiency of lipospermine/DNA particles" in *Gene Therapy* 5(6): 855-860, 1998.)

Claim 12 refers to cholesterols, which one of ordinary skill in the art would understand to mean cholesterol and derivatives thereof. For example, in Example 8 on page 45, line 28, the specification teaches the conjugation of cholesterol to hydrophilic polysaccharides. In addition, on pages 23 and 26, the specification discloses a number of derivatives of cholesterol, including Cholesteryl chloroformate, Cholesteric acid N-hydroxy succinimide (NHS) ester, di-Chol-L-Lysine-NHS, and di-Chol-L-Lysine-OMe. Furthermore, a number of papers disclosing cholesterol derivatives were published well before the filing of the current application (see, for example, the enclosed copies of Farhood H et al., "Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity" in *Biochim Biophys Acta.* 1111(2):239-46 (1992) and Vigneron JP et al., "Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells" in *Proc. Natl. Acad. Sci. USA* 93(18):9682-6 (1996)).

Another reason why claims 10 and 12 should not be rejected is because the breadth of a claim is not to be equated with indefiniteness (*see re Miller*, 441 F.2d 689, 169 USPQ 597 (CCPA 1971)). Section 2173.04 of the MPEP reads, "If the scope

of the subject matter embraced by the claims is clear, and if applicants have not otherwise indicated that they intend the invention to be of a scope different from that defined in the claims, then the claims comply with 35 U.S.C. § 112, second paragraph." Applicant submits that the scope of claims 10 and 12 is clear and correctly defines the scope of the invention.

Claim 23 has been amended to refer to a porous matrix suitable for use as a scaffold for cell growth, and claim 24 has been amended to specify a cationic coating composition suitable for use in the printing or electronic industries. Support for these amendments can be found in the specification on page 54, lines 18-22 (claim 23) and page 10, line 36 (claim 24).

In summary, applicant submits that the amended claims now comply with U.S.C. § 112, second paragraph if examined in light of the requirements for analyzing the definiteness of claim language.

Allowance of all claims, including 8, 10, 12, 23 and 24, as amended, is respectfully solicited.

Respectfully submitted,

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Genetics

Guanidinium-cholesterol cationic lipids: Efficient vectors for the transfection of eukaryotic cells

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Contributed by Jean-Marie Lehn, March 8, 1996

ABSTRACT **Two cationic lipids, bis-guanidinium-spermidine-cholesterol (BGSC) and bis-guanidinium-tren-cholesterol (BGTC)—cholesterol derivatives bearing two guanidinium groups—have been synthesized and tested as artificial vectors for gene transfer. They combine the membrane compatible features of the cholesterol subunit and the favorable structural and high pK_a features of the guanidinium functions for binding DNA via its phosphate groups. Reagent BGTC is very efficient for transfection into a variety of mammalian cell lines when used as a micellar solution. In addition, both BGTC and BGSC present also a high transfection activity when formulated as liposomes with the neutral phospholipid dioleoylphosphatidyl ethanolamine. These results reveal the usefulness of cholesterol derivatives bearing guanidinium groups for gene transfer.**

Synthetic nonviral vectors represent an attractive alternative approach to viral vectors for gene transfer studies and gene therapy applications. Various cationic lipids have been shown to induce efficient transfection of a large variety of eukaryotic cells. Most of them in use today are formulated as liposomes containing two lipid species, a cationic amphiphile and a neutral phospholipid, typically dioleoylphosphatidyl ethanolamine (DOPE) (1). However, some lipids (e.g. lipopolyamines) can be used directly as cationic amphiphilic reagents in solution (2). The spontaneous formation of DNA/lipid aggregates *in vitro* is in any case due to ionic interactions between the positively charged cationic lipid and the negatively charged phosphate groups of the DNA; residual positive charges on the aggregates presumably mediate their binding to negatively charged (sialic acid) residues on cell surfaces.

3β[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), a cationic cholesterol derivative, can be used in combination with DOPE to prepare liposomes that have been shown to efficiently transfect mammalian cells (3). These DC-Chol/DOPE liposomes have even already been used in gene therapy applications in the clinical setting (4, 5).

We have thus directed our efforts toward the design of cationic cholesterol derivatives that would be characterized by novel cationic moieties and by their solubility behavior in aqueous phase. Although such transfection reagents could be used to prepare cationic liposomes, they should also be efficient directly as aqueous (micellar) solutions, avoiding thereby the need to prepare and the variability associated with cationic liposomes.

The guanidinium group appeared particularly well suited for interaction with the phosphate residues of polynucleotides with which it is able to establish a characteristic pair of hydrogen bonds. We report here the synthesis of bis-guanidinium cholesterol lipids and their use for the efficient transfection of various mammalian cell lines as water soluble reagents as well as in the form of cationic liposomes. Our data demonstrate the usefulness, convenience, and versatility of cationic cholesterol derivatives with guanidinium polar head groups for gene transfer.

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MATERIALS AND METHODS

Synthesis of Bis-Guanidinium-Cholesterol Derivatives: Bis-Guanidinium-Spermidine-Cholesterol (BGSC) and Bis-Guanidinium-Tren-Cholesterol (BGTC). *Materials.* Spermidine, cholesteryl chloroformate, and tris(2-aminoethyl)amine (TREN) were obtained from Aldrich and were used as supplied. $^1N,^8N$-Boc_2-spermidine (6) and 1*H*-pyrazole-1-carboxamidine hydrochloride (7) were prepared according to published procedures. All the solvents were analytical reagent grade and were used without further purification.

Synthesis of BGSC. BGSC (3β[^8N-($^1N,^8N$-diguanidino spermidine)-carbamoyl]cholesterol) was prepared as outlined in Fig. 1. $^1N,^8N$-Boc_2-spermidine (6) was reacted with cholesteryl chloroformate in CH_2Cl_2 in presence of triethylamine. The resulting compound was deprotected by trifluoroacetic acid in CH_2Cl_2, and the free diamine was obtained by aqueous NaOH treatment. Guanylation of this diamine was effected by reaction with 1*H*-pyrazole-1-carboxamidine hydrochloride (7) in presence of diisopropylethylamine. Slow diffusion of diethylether in a methanolic solution of this crude product yielded pure BGSC (61%) as judged by 200 MHz ^{1}H NMR (model AC 200; Bruker, Wissembourg, France). δ(dimethyl sulfoxide *d6*): 0.5–2.5(*m*, cholesteryl skeleton and central CH_2), 3.1(*m*, 8H, *N*-CH_2), 4.3(*m*, 1H, $H_{3\alpha}$), 5.3(*d*, 1H, H_6), 7.2(broad *s*, 8H, NH_2^+), 7.8(*s*, 2H, NH). Analysis calculated for $C_{37}H_{69}N_7O_2Cl_2$: *C*, 62.15; H, 9.67; N, 13.71. Found: *C*, 62.28; H, 9.81; N, 13.15. (Service Régional de Microanalyse, Université Paris 6).

Synthesis of BGTC. The reaction scheme for the synthesis of BGTC, (3β[*N'*,*N'*-diguanidinoethyl-aminoethane)carbamoyl] cholesterol), is given in Fig. 1. A large excess of TREN was reacted with cholesteryl chloroformate in CH_2Cl_2. The unreacted amine was removed by washing with water, and the crude product was dissolved in tetrahydrofuran/MeOH (50:50). To this mixture was added 2 equivalents of 1*H*-pyrazole-1 carboxamidine (7) and 2 equivalents of diisopropylethylamine. After stirring at room temperature for 18 h, diethylether was added and the resulting precipitate was separated by decantation. To get a pure sample, this crude compound was suspended three times in diethylether and separated by decantation (60% after drying under vacuum). ^{1}H NMR(dim-

Abbreviations: DOPE, dioleoylphosphatidyl ethanolamine; DC-Chol, 3β[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol; BGSC, bis-guanidinium-spermidine-cholesterol; BGTC, bis-guanidinium-tren-cholesterol; TREN, tris(2-aminoethyl)amine; RLU, relative light units.

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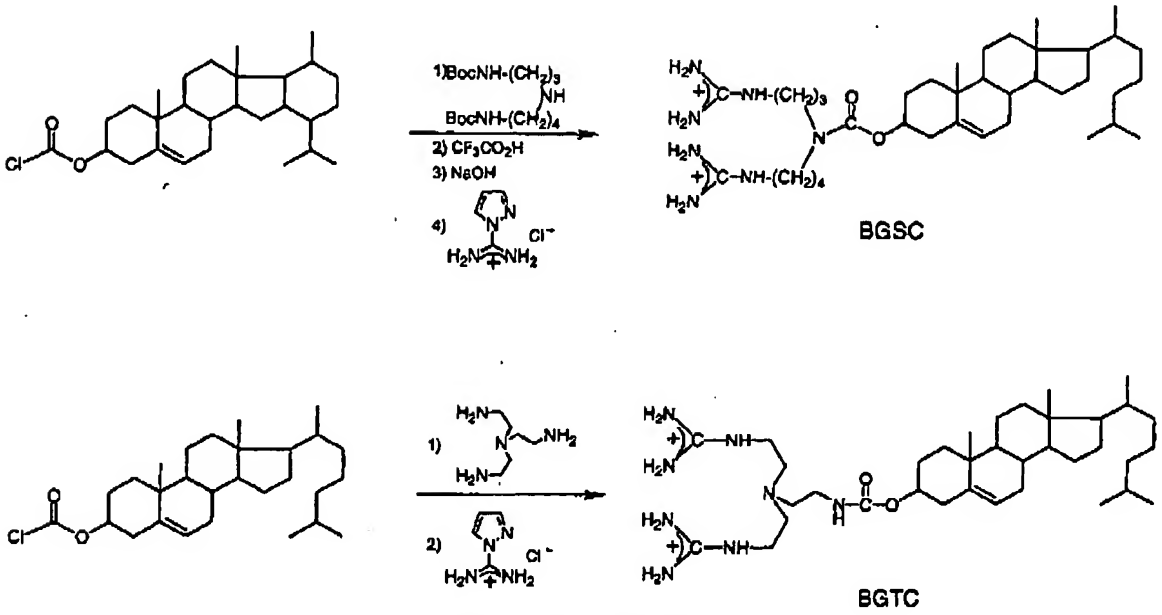

FIG. 1. Scheme for the synthesis of BGSC and BGTC.

ethyl sulfoxide d_6, 200 MHz): 0.5–2(*m*, cholesteryl skeleton), 2.2(*m*, 2H, N-CH_2), 2.6(broad *s*, 4H, N-CH_2), 3(*d*, 2H, N-CH_2), 3.2(broad *s*, 4H), 4.3(*m*, 1H, $H_{3\alpha}$), 5.3(*d*, 1H, H_6), 7.3(broad *s*, 8H, NH_2^+), 7.8(broad *s*, 2H, NH). Analysis calculated for $C_{36}H_{68}N_8O_2Cl_2$: *C*, 60.39; H, 9.57; N, 15.65. Found: *C*, 60.38; H, 9.67; N, 15.56 (Service Régional de Microanalyse, Université Paris 6).

Liposome Preparation. A mixture of cationic lipid and DOPE (molar ratio 3:2) in $CHCl_3$ was evaporated under vacuum and resuspended under N_2 atmosphere in a 20 mM Hepes buffer solution (pH 7.4). The final lipid concentration was 1.2 mg/ml. The mixture was vortex mixed for 5 min, then sonicated for 5 min in a bath sonicator (Branson Ultrasonics 2210) and stored at 4°C for 24 h for hydration. The resulting dispersion was sonicated again (Branson Sonifier 450) for 5–10 min to form liposomes. After centrifugation, the solution was filtered through a 0.22 μ filter (Millex GS; Millipore) and stored at 4°C. For both BGSC- and BGTC-containing liposomes, liposome size distribution was studied by using a laser light scattering apparatus (Autosizer 4700; Malvern Instruments, Orsay, France) and showed an unique peak corresponding to 50 nm in average diameter by multimodal analysis by number.

Cell Culture. Species and tissue specificities of most of the cell lines used for transfection experiments are indicated in Table 1. The other cell lines tested were as follows. HeLa cells (P. Briand, Institut Cochin de Génétique Moléculaire, Paris) are derived from a human epithelioid cervical carcinoma. NIH 3T3 cells (C. Lagrou, Institut Pasteur, Lille, France) are mouse fibroblasts. The NB 2A cell line (C. Gouget, Paris) is derived from a mouse neuroblastoma.

All cells, except AtT-20 and PC 12 cells, were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (FCS; GIBCO), penicillin at 100 units/ml (GIBCO), and streptomycin (GIBCO) at 100 μg/ml. AtT-20 cells were propagated in DMEM/F12 (GIBCO) supplemented with 10% FCS, and PC12 cells were grown in DMEM supplemented with 10% FCS and 5% horse serum. All cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37°C in a humidified 5% CO_2/95% air containing atmosphere.

Plasmids. Plasmid pRSV-Luc (O. Bensaude, Ecole Normale Supérieure, Paris), where the luciferase reporter gene is

Table 1. Luciferase expression in various mammalian cell lines transfected with either lipid BGTC, calcium phosphate, or Transfectam

			RLU/mg of cell protein		
Species	Cell line	Tissue	BGTC lipid	Calcium phosphate	Transfectam
Human	A549	Lung carcinoma	4×10^5	7×10^3	3.1×10^5
Monkey	COS-7	Simian virus-40 transformed kidney	2.1×10^7	3.7×10^5	8.4×10^6
Dog	MDCK-1	Kidney	3×10^6	4.5×10^5	2.2×10^6
Rat	RIN-m5F	Pancreatic islet cell tumor	2×10^7	2×10^5	3.3×10^5
	ROS	Osteosarcoma	4.0×10^6	4.5×10^5	2.3×10^6
	PC12	Pheochromocytoma	3×10^7	1.7×10^5	6×10^6
Mouse	AtT-20	Pituitary tumor	2×10^7	8×10^5	3×10^7

Transfections and luciferase assays were carried out as described. All transfections were performed at least in duplicate ($n \geq 2$) and data for luciferase activity are expressed as mean relative light units normalized to 1 mg of cell protein (RLU/mg protein).

under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat, was amplified in *Escherichia coli* and prepared by CsCl gradient purification using standard techniques.

Transfection Procedure. A general protocol for typical transfection was as follows. Cells from various cell lines were seeded at about 2×10^5 cells per well in 6-well dishes (Falcon) the day before transfection in order to be approximately half-confluent the next day.

Plasmid DNA (5 μg) and the desired amount of bis-guanidinium lipid were each diluted into 250 μl of DMEM without FCS and vortex mixed. After ≈5 min, the two solutions were mixed and the resulting solution was allowed to incubate about 15 min at room temperature. The transfection mixture was then added (0.5 ml per well) to the cells which had been washed with serum-free medium. After 4–6 hr incubation at 37°C, 1 ml of serum-containing medium was then added per well without removing the transfection mixture. Twenty-four hours after transfection, the medium was replaced with 1 ml of fresh culture medium. The cells were harvested at 2 days posttransfection for monitoring of the transient expression of the luciferase gene.

Control transfections were performed by using widely available transfection reagents. The lipopolyamine Transfectam (a gift from J. P. Behr, Strasbourg, France) was used as an ethanolic solution and at an optimal Transfectam/DNA ionic charge ratio of 6–8 (8). For transfection with the commercial reagent Lipofectin (Life Technologies, Cergy Pontoise, France), which is a liposome formulation of the cationic lipid *N*[1-(2, 3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and DOPE, DNA complexes were obtained by using standard conditions recommended by the manufacturer. Cells were transfected with calcium phosphate-precipitated DNA as described (9).

Luciferase Assay. Luciferase activity was assayed 48 h after transfection using a variation of the procedure of De Wet *et al.* (10). After removal of the culture medium, the cells were washed with cold phosphate-buffered saline and lysed by incubation with 250 μl lysis buffer (25 mM triphosphate, pH 7.8/8 mM $MgCl_2$/1 mM dithiothreitol/15% glycerol/1% Triton X-100). The lysate was clarified from insoluble material by centrifugation (for 15 min at 4°C) in a microcentrifuge. An aliquot (20 μl) of cell extract was diluted in 100 μl of lysis buffer, to which 4 μl of 25 mM ATP (Sigma) and 20 μl of 25 mM luciferin (Sigma) were then added. Samples were placed in a photon-counting luminometer (Lumet LB 9501; Berthold, Nashua, NH) and the integration value of the light emission for 10 s was measured. A standard curve of relative light units (RLU) versus luciferase was made by using a dilution series of purified firefly luciferase (Sigma) and showed that the linear range of light detection extends from 10^4 to 10^7 RLU (data not shown). Protein concentrations were measured by the bicinchoninic acid assay (Pierce) using bovine serum albumin as standard. Data for luciferase activity are expressed as relative light units per mg of cell proteins (RLU/mg protein).

RESULTS AND DISCUSSION

Synthesis and Features of Bis-Guanidinium Transfection Reagents. The two new cationic lipids, BGSC and BGTC, were obtained in good yields by a straightforward synthesis requiring inexpensive reagents (Fig. 1). They are cholesterol derivatives bearing guanidinium groups. On one hand, the cholesterol unit has been shown to facilitate the cellular uptake of various oligonucleotides (11, 12) and polar drugs (13). On the other hand, the guanidinium group presents several interesting features: (*i*) it remains protonated over a much wider range of pH than the ammonium group, due to its much higher pK_a [13.5 for guanidinium itself (14)]; (*ii*) it forms with phosphate anions, characteristic pairs of parallel zwitterionic hydrogen bonds $N\text{-}H^+ \cdots O^-$, which provide binding strength by their charge and structural organization, as seen for instance in the crystal structure of methylguanidinium dihydrogen phosphate (15); (*iii*) the guanidinium group is also able to develop hydrogen bonding with nucleic bases, especially with guanine (16, 17); (*iv*) finally the guanidinium group of arginyl residues has a major function in DNA-binding proteins, histones and protamines.

Due to the high pK_a of the guanidinium group, the transfection efficiency of the lipids BGSC and BGTC should be relatively insensitive to variations of pH during the *in vitro* formation of the DNA/reagent aggregates and the trafficking in the cell toward the nucleus. Furthermore, the tertiary amine of BGTC, which is situated between two positive guanidinium groups and has probably a lower pK_a, could also be able to buffer the acidic environment of late endosomes and of lysosomes, thus protecting the DNA against degradation as recently suggested for the cationic polymer polyethylenimine (18).

To obtain some information about the nature of the species formed by these cationic lipids in aqueous phase, surface tension depression measurements were conducted. They indicated that compound BGTC gives true micellar solutions in the concentration range used in the transfection experiments, with a critic micellar concentration value of 9×10^{-5} M.

Transfection Activity of the BGTC Reagent in Aqueous Medium. As compound BGTC was found to form true micellar solutions (see above), we investigated its potential usefulness as transfection reagent by direct mixing of its solution with the plasmid-containing solution. Previous *in vitro* studies (1–3) suggested that cationic lipid-mediated gene transfer is efficient when the lipid/DNA aggregates formed have a net positive charge that can associate with anionic residues on the cell membrane. To optimize the BGTC lipid/DNA aggregates formation for transfection, we first examined the influence of the BGTC lipid/DNA ratio on transient transfection activity in three different types of mammalian cells known to be relatively easy to transfect with classical techniques.

Transfection experiments with murine 3T3 fibroblasts, human epithelial HeLa cells, and the mouse neuroblastoma NB2A cell line were performed by mixing a fixed amount of plasmid pRSV-Luc with various amounts of compound BGTC in solution, as indicated in *Materials and Methods*. To determine the range of the BGTC/DNA ratio to be screened, we took into account that 1 μg of DNA is 3 nmol of negatively charged phosphate and assumed, as discussed above, that only the two guanidinium groups (and not the tertiary amine) of BGTC are positively charged at the neutral pH of DNA aggregates formation and of transfection. Transfection results with the three cell lines are indicated in Fig. 2, where luciferase reporter gene expression at 48 hr posttransfection is shown as a function of the BGTC guanidinium/DNA phosphate ratio. Fig. 2 shows that the ratio of BGTC lipid to DNA is critical to the reagent's efficacy. Luciferase expression was highest when working with aggregates containing ≈6 (HeLa cells) to ≈8 (NB2A cells and 3T3 cells) guanidinium groups per DNA phosphate—i.e., aggregates bearing a strong positive charge. Lower guanidinium/phosphate ratios were less efficient and, at very high ratios, there was a decline in transfection activity associated with a reduction in the number of cells per well (Fig. 2 and data not shown). Thus, the BGTC lipid-dose response curves at fixed DNA were roughly bell-shaped (Fig. 2). Such bell-shaped curves have already been described by various investigators using different types of cationic lipids (1, 3). It is generally agreed that the low efficiency of low lipid/DNA ratios is due to reduced cell membrane binding, and that the decline in transfection activity at high ratios reflects toxic effects of the cationic lipid.

Cationic lipid-mediated gene transfer has become an attractive transfection technique because it is not only straightforward but it can also be applied to a wide variety of cells of

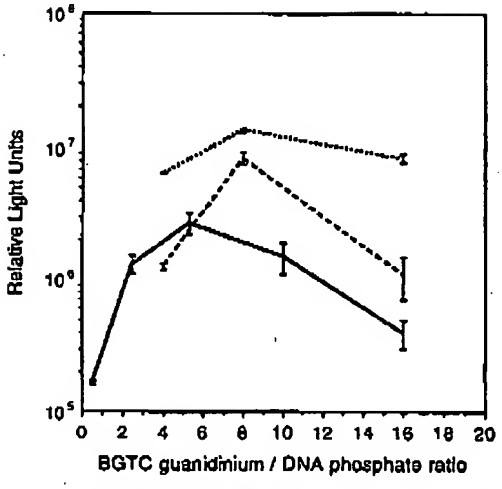

FIG. 2. Luciferase reporter gene expression as a function of the mean ionic charge of the lipid/DNA aggregates (BGTC guanidinium to DNA phosphate ratio). HeLa cells (——), NB2A cells (···), and NIH 3T3 cells (– – –) were transfected as described by using a fixed amount of DNA with various amounts of BGTC lipid. Data are expressed as mean RLU (± SD) ($n \geq 3$)

different species and tissues. To investigate the spectrum of effectiveness of the BGTC reagent for transient transfection of mammalian cells, we performed transfections with a variety of cell lines routinely used in research laboratories, including cells (e.g., PC12 cells) known to be difficult to transfect by conventional techniques. In these experiments, we used a standard guanidinium/phosphate ratio of 6–8, as this ratio was shown to be optimal by the lipid-dose response curves (see above). In addition, for comparative purposes, the various cell lines tested were also transfected, as indicated in *Materials and Methods*, by the classical calcium phosphate precipitation method and by the lipopolyamine Transfectam, a commercially available cationic lipid very efficient for transferring genes into eukaryotic cells *in vitro* and which is also used as a solution without liposomal formulation. The results are listed in Table 1. They show that lipid BGTC is usually as efficient as Transfectam and is in general 1 to 2 orders of magnitude more efficient than the calcium phosphate technique. Thus, we could satisfactorily transfect all the cell lines screened using non toxic levels of BGTC. There was some variability in transfection efficiencies between the different cell lines, but some cell lines are intrinsically more difficult to transfect and, in addition, the transfection conditions should probably be worked out for each individual cell type for optimal results.

Our data demonstrate that compound BGTC, a cationic cholesterol derivative with guanidinium polar groups, is an efficient reagent for transient transfection of various cell types. It can be used without liposomal formulation and is efficient despite the fact that it contains neither the fusogenic lipid DOPE, in contrast to the cationic liposomes DC-Chol/DOPE, nor two fatty acid chains, unlike many cationic lipids. However, evaluation of cationic lipids using expression of a transgene as the end point is a very empirical approach. In particular, complex formation, cell binding, and intracellular trafficking were not examined here. It will also be interesting to study the transfection efficiency of reagent BGTC for stable transfection and for transfection of cells growing in suspension.

Transfection Activity of Compounds BGSC and BGTC Formulated as Cationic Liposomes with DOPE. Because the synthetic compound BGSC is less soluble in aqueous medium than BGTC, we have evaluated its potential for transfection using a liposome formulation with DOPE (BGSC/DOPE molar ratio, 3:2). In addition, because many cationic lipids are formulated as liposomes with DOPE, we have also prepared BGTC/DOPE (3:2) liposomes following the procedure indicated in *Materials and Methods*.

As shown above for BGTC used as an aqueous solution, and because cationic liposome-mediated *in vitro* transfection has also been shown to be more efficient when lipid/DNA aggregates bear a net positive charge, we first performed transfection experiments with HeLa cells to establish a lipid-dose response curve (at a fixed amount of plasmid DNA) in order to determine the optimal lipid to DNA ratio of the BGSC liposomes/DNA aggregates. As shown in Fig. 3, the transfection activity of the BGSC—DOPE liposome reagent displayed a bell-shaped dependence on the lipid concentration, with a broad maximum characterized by a BGSC guanidinium/DNA phosphate ratio centered around 2.5–3 (range 1.5–5). Thus, since the guanidinium groups of compound BGSC are protonated at neutral pH, the optimal BGSC-DOPE/DNA aggregates have a net positive charge of ~3. It appears that this optimal lipid guanidinium/DNA phosphate ratio of BGSC-DOPE/DNA aggregates can be lower than that of optimal BGTC/DNA complexes, which were found to have a ratio of ~6–8 (see above). This observation could support the role of a DOPE-facilitated step in cationic liposome-mediated transfection, involving in particular the well-known fusogenic properties of DOPE. Indeed, it has been shown that, for a series of cationic lipid molecules, formulations with 50 mol % DOPE were 2- to 5-fold more active than formulations without any zwitterionic lipid (1). Our observation also invites the exploration of the adjuvant effect of other additives.

To further assess the convenience and the versatility of compounds BGSC and BGTC, we have transfected various adherent cell lines using liposome formulations with DOPE (molar ratio, 3:2). For these experiments, we used a lipid guanidinium/DNA phosphate ratio of 2.5 (~3) for both gene transfer systems. Control transfection experiments were also performed with the Lipofectin reagent, as indicated in *Materials and Methods*. Transfection data are indicated in Table 2. The results show that both BGSC and BGTC liposomes gave

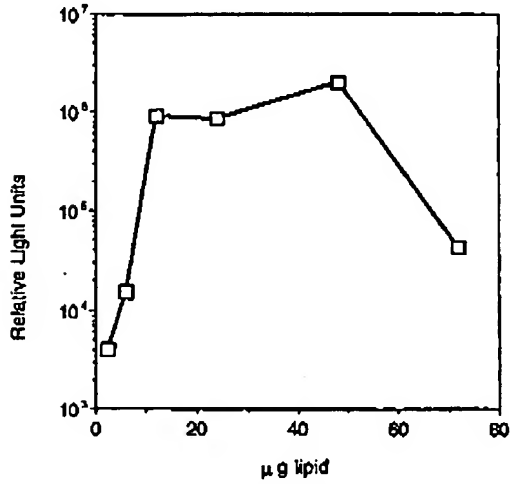

FIG. 3. Transfection activity of liposomes composed of BGSC and DOPE (3:2) in HeLa cells. Transfections were performed as described by using a fixed amount of DNA (5 μg) with various amounts of BGSC–DOPE liposomes. Data are expressed as mean RLU/mg of cell protein ($n \geq 2$).

Table 2. Luciferase expression in various eukaryotic cell lines transfected by BGSC–DOPE liposomes, by BGTC–DOPE liposomes, and by Lipofectin reagent

	RLU/mg of cell protein		
Cell line	BGSC liposome	BGTC liposome	Lipofectin
HeLa	4.6×10^6	7.7×10^6	3.3×10^6
A 549	6×10^5	2×10^5	4×10^6
COS-7	ND	1.4×10^7	9.5×10^6
MDCK-1	1×10^6	7×10^6	1.9×10^6
ROS	ND	9×10^6	6×10^6
NB2 A*	1.5×10^7	1.4×10^7	ND
NIH 3T3*	7×10^6	1.5×10^6	ND

Transfections and luciferase assays were carried out as described. All transfections were performed at least in triplicate ($n \geq 3$), and luciferase activity is expressed as mean relative light units per mg of cell protein (RLU/mg protein). ND: not determined.
*Mean RLU of luciferase measurements on 100 μl/500 μl total lysate ($n \geq 4$).

satisfactory transfection of all the cell lines tested. Their efficacy is quite similar and appears also to be similar to that of the Lipofectin reagent. However, as already discussed above, for a more precise comparison, the various steps of the transfection protocol should be optimized for each cell type. Thus, these data demonstrate that bis-guanidinium cholesterol derivatives can also be used as liposome formulations with DOPE for efficient *in vitro* transient transfection.

In conclusion, our results clearly show that cholesterol derivatives with guanidinium polar head groups are efficient and convenient reagents for gene transfer into a wide variety of mammalian cell lines. Interestingly, compound BGTC can be used as an aqueous solution, thereby avoiding the preparation of cationic liposomes. Studies are in progress to further explore the usefulness of such transfection reagents (for *in vivo* transfection) and to design improved vectors based on a guanidinium cholesterol-DNA core.

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Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity.

Farhood H, Bottega R, Epand RM, Huang L.

Cell, Molecular and Developmental Biology Program, University of Tennessee, Knoxville.

Four different cationic derivatives of cholesterol were synthesized which contain either a tertiary or a quaternary amino head group, with and without a succinyl spacer-arm. Their ability to inhibit protein kinase C (PKC) activity was measured in a detergent mixed micellar solution. Derivatives containing quaternary amino head group were effective inhibitors (Ki approx. 12 and 59 microM) of PKC and derivatives containing a tertiary amino head group were approx. 4-20-fold less inhibitory. Liposomes containing an equimolar mixture of dioleoylphosphatidylethanolamine (DOPE) and a cationic cholesterol derivative were tested for the DNA-mediated transfection activity in mouse L929 cells. Highest activity was found with the derivative with low PKC inhibitory activity and with a succinyl spacer-arm. The transfection activity of this tertiary amine derivative, N,N-dimethylethylenediaminyl succinyl cholesterol was dependent on DOPE as a helper lipid; liposomes containing dioleoylphosphatidylcholine and this derivative had little activity. The transfection protocol of this new cationic liposome reagent was optimized with respect to the ratio of liposome/DNA, dose of the complex and time of incubation with cells. Several adherent cell lines could be efficiently transfected with this liposome reagent without any apparent cytotoxicity. However, the transfection activity was strongly inhibited by the presence of serum components.

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BRIEF COMMUNICATION

Influence of the DNA complexation medium on the transfection efficiency of lipospermine/DNA particles

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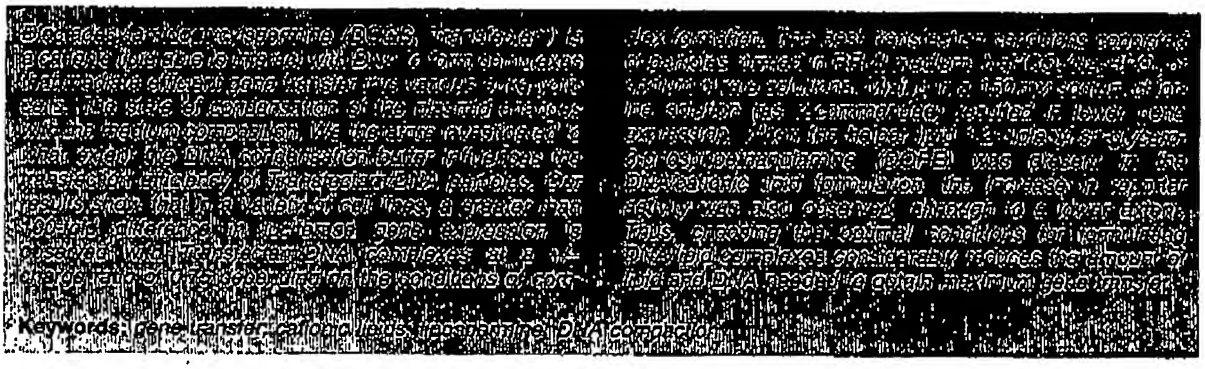

Keywords: gene transfer; cationic lipid; lipospermine; DNA/lipoplex

The development of highly efficient gene transfer methods is a prerequisite for gene therapy. One of the approaches involves artificial self-assembling systems. Typically, (poly)cationic molecules interact with DNA to form particles that are able to deliver the DNA into the cells. Cationic lipids are some of the more promising vectors.[1–4] However, it is estimated that only one out of 10^4 plasmid molecules presented to the cell by cationic lipids, reaches the nucleus and is expressed. In contrast, for every 10 recombinant adenoviruses that reach a cell, at least one will successfully transfer and express its gene.[5] Efforts to improve the lipofection (gene transfer using cationic lipids[6]) efficiency include direct modification of the cationic lipid,[7,8] addition of helper molecules such as the lipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine),[9] addition of replication-defective adenoviruses[10] or fusigenic peptides.[11,12] Despite encouraging advances, the number of plasmid copies required to obtain high gene expression is still high.

Cationic lipids with multivalent headgroups, such as lipospermines, are usually more efficient for *in vitro* gene transfer than the monovalent cationic lipids.[13] However, transfection with these lipids *in vitro* requires DNA complexes bearing a strong net positive charge, ie in previous studies using Transfectam (dioctadecylamidoglycylspermine, DOGS), it was found that the transfection efficiency was optimal when the +/− charge ratio was about 3 to 6.[14] These positive charges are thought to promote binding and uptake of the complexes through nonspecific endocytosis.[15,16] However, the low gene transfer efficiency observed *in vivo* with synthetic vectors may be due to the interactions of the positively charged transfection particles with blood components. In contrast, lipoplexes (cationic lipid/DNA complexes[6]) with charge ratios close to neutrality should allow: (1) a reduced binding to the anionic extracellular matrix; and (2) a reduced activation of the complement system.[17] In addition, there is a dose-dependent toxicity associated with the use of lipoplexes. Thus, it is necessary to reduce the quantity of material (ie the DNA and its carrier) required to achieve efficient gene delivery *in vivo*.

We report that the optimization of the DNA/Transfectam condensation step using suboptimal amounts of cationic lipid leads to highly efficient formulations.

Complexes of DNA and 2, 1 and 0.75 charge equivalents of Transfectam were prepared (the term charge equivalent indicates the amount of lipid used for the formation of the complexes; one charge equivalent corresponds to the amount required to neutralize all the negative charges carried by the phosphate groups of the plasmid; the charge ratio is calculated by taking into account the fact that one molecule of Transfectam carries three positive charges, as three ammonium groups are protonated at physiological pH[13]). When formulated in Roswell Park Memorial Institute (RPMI; Gibco-BRL, Gaithersburg, MD, USA) culture medium instead of 150 mM NaCl solution as recommended by the manufacturer, these lipoplexes gave a 600, 250 and 200 times higher luciferase reporter gene expression (Figure 1a), thus reaching the values obtained with highly positively charged complexes (compare with Transfectam

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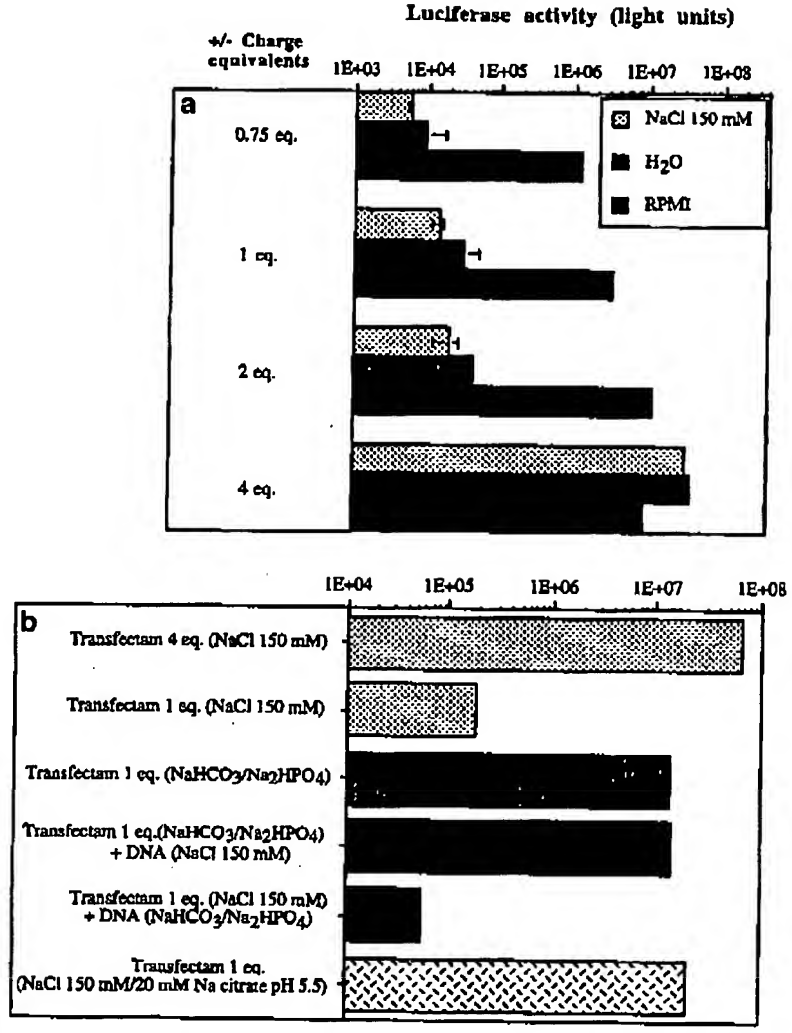

Figure 1 Influence of the buffer composition used for the DNA condensation step on reporter gene expression. The plasmid coding for the Photinus pyralis luciferase gene under control of the cytomegalovirus enhancer/promoter[18] (pCMVL) was used. Complexes were prepared as follows: plasmid DNA (3 or 4 μg) and the desired amount of cationic compound were each diluted into 75 μl of buffer (as indicated in the Figures) and gently mixed. After about 15 min, the two solutions were mixed. After 10 more min, the mixture was diluted with serum-free medium to a final volume of 1.5 ml; 750 μl of the transfection mixture was put on each well of the duplicate. (a) Complexes of 0.75, 1, 2 and 4 charge equivalents of Transfectam/3 μg pCMVL prepared in 150 mM NaCl, H_2O or RPMI were used. The DNA complexes were finally mixed with RPMI medium and added to the human melanoma cells H225 (75 000 cells per well in 24-well plates; the cells were kindly provided by S Schreiber and G Stingl, University of Vienna, Austria). After 4 h, the transfection medium was replaced by fresh RPMI supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal calf serum (FCS; Gibco-BRL). The cells were lysed after 24 h in 200 μl of 250 mM Tris pH 7.3, 0.5% Triton X-100. The cell lysate was centrifuged for 5 min at 10 000 g to pellet debris. Luciferase light units were recorded using a Clinilumat LB9502 instrument (Berthold, Bad Wildbad, Germany) from an aliquot of the supernatant with 10 s integration. Luciferase background (approximately 300 light units) was subtracted from each value and the transfection efficiency was expressed as total light units per well and represents the mean value of the duplicates (±s.d.). (b) Determination of the RPMI component(s) responsible for the increased transgene expression. Transfectam used at 1 charge equivalent was complexed with 3 μg DNA (per duplicate) in either 150 mM NaCl, NaCl/Na citrate (pH 5.5), or $NaHCO_3/Na_2HPO_4$. For the remaining formulations, the cationic lipid pre-incubated in $NaHCO_3/Na_2HPO_4$ (or NaCl), was mixed with the DNA diluted in NaCl (or $NaHCO_3/Na_2HPO_4$). The efficiencies of these formulations were compared to Transfectam 4 eq/DNA complexes prepared in NaCl 0.15 M.

4 eq/DNA prepared in 150 mM NaCl, which leads to the highest luciferase activity on H225 cells). By replacing the RPMI medium with DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL), the enhancement observed with suboptimal amounts of lipid (0.75, 1 and 2 eq) was less significant, but the efficiency remained better than with water or 150 mM NaCl (not shown). In contrast, when optimized amounts of cationic lipid, ie 4 charge equivalents, and DNA (2 μg per well for a 24-well plate) were mixed in RPMI, there was no enhancement in reporter gene expression. In fact, a decrease f the efficiency was observed, which is consistent with the data obtained by Barthel and colleagues.[14]

In order to determine which components are respon-

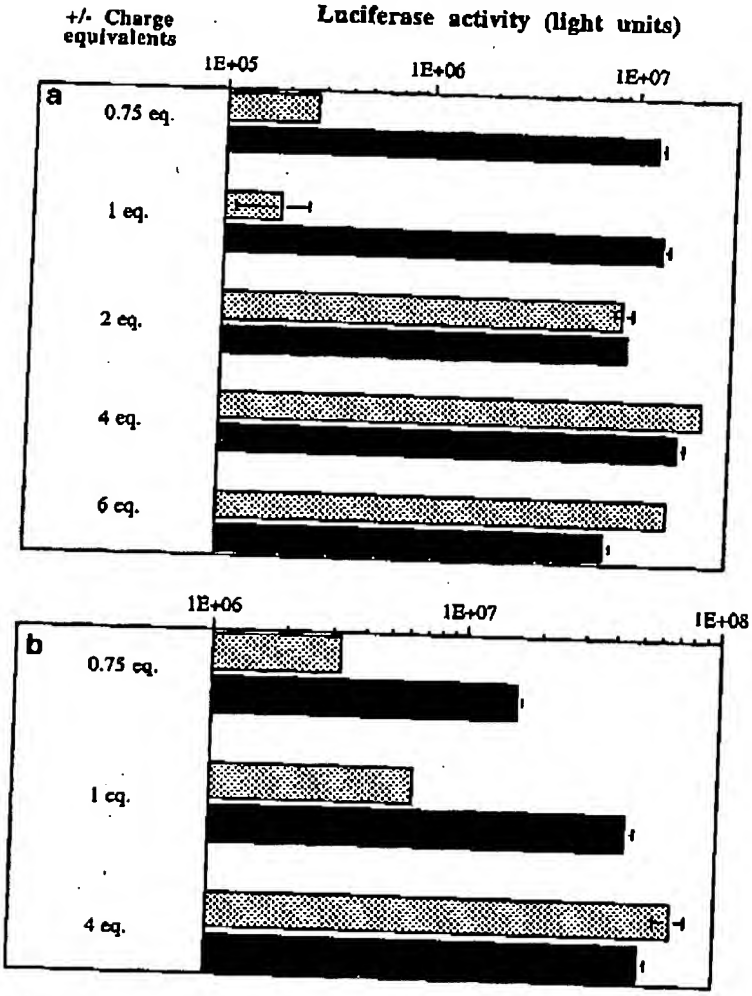

Figure 2 The enhancement of reporter gene expression can be observed on several cell types. The efficiencies of different lipoplexes, prepared in NaCl (light bars) or in $NaHCO_3/Na_2HPO_4$ (dark bars), were determined on (a) HepG2 cells (120 000 cells per well were plated on the day before transfection in 24 well-plates) and (b) B16 cells (70 000 cells per well): both cell types were cultured in DMEM supplemented with antibiotics, glutamine and 10% heat-inactivated FCS. The transfection experiments were carried out as indicated in Figure 1. Total luciferase activity of the cells is shown and represents the mean value of the duplicates (±s.d.).

sible for the increased efficiency with suboptimal amounts of lipid, we tested each salt, alone or in combination with other components of the RPMI medium. $NaHCO_3$ (23.8 mM; pH 7.4) and Na_2HPO_4 (5.6 mM; pH 7.4) used alone (data not shown) or mixed together (pH 8; Figure 1b) gave a similar enhancement to that of the complete culture medium. In addition, we tested other buffers for their ability to augment the efficiency of 1 charge equivalent Transfectam/DNA complexes; a buffer containing 150 mM NaCl and 20 mM sodium citrate (pH 5.5) was as efficient as $NaHCO_3/Na_2HPO_4$ (Figure 1b). Thus, in contrast to the medium effect observed by Caplen *et al*[19] with the cationic formulation DC-Chol/DOPE, the effect we observed is not pH-dependent (ie it does not rely on a modified protonation state of the amines of the lipospermine).

Using the lipoplexes prepared in the $NaHCO_3/Na_2HPO_4$ buffer on other cell lines, such as the human hepatocarcinoma cell line HepG2, B16 murine melanoma cells or the murine fibroblasts NIH 3T3, comparable results to those observed on H225 cells were obtained (Figure 2, and Otmane Boussif, personal communication), showing that the effect is not cell type-dependent. An enhancement, although to a lower extent, was observed using Lipofectamine[20] (Gibco-BRL) under suboptimal conditions (0.75 eq; see Figure 3). Lipofectamine is a mixture of the lipospermine DOSPA (2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate) and the helper lipid DOPE (DOSPA/DOPE, 3/1, w/w). It was previously shown that DOPE can increase the efficiency of Transfectam when suboptimal amounts of lipid are used.[9] No enhancement in rep rter gene expression was obtained with Transfectam 2 eq/DOPE 1.5 eq/DNA particles. These results confirm the Lipofectamine data, demonstrating that the presence of DOPE diminishes the

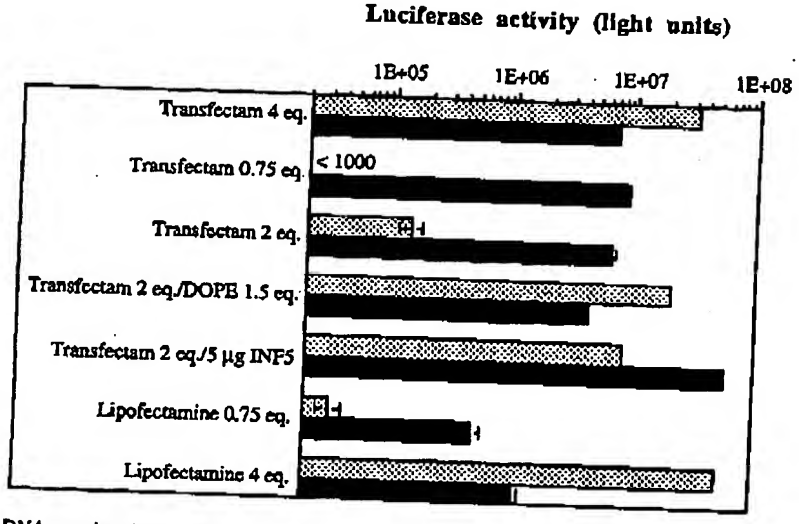

Figure 3 Influence of the DNA complexation medium on the efficiency of different lipospermine-based formulations. Transfectam- and Lipofectamine-based complexes were prepared (as described in Figure 1) either in 0.15 M NaCl (light bars) or in a $NaHCO_3/Na_2HPO_4$ solution (dark bars). The Transfectam/DOPE complex was obtained by mixing the desired amounts of Transfectam and helper lipid DOPE (the amount of helper lipid used is given in equivalents (mol/mol) to Transfectam) before dilution with the DNA solution. To obtain the peptide formulation, the fusigenic peptide INF5[21] (1 mg/ml solution in HBS/glycerol (3:1)) was added to the preformed Transfectam/DNA complexes; after a 10 to 20 min period, the transfection volume was adjusted to 1.5 ml with culture medium, and 750 µl of this transfection mixture per well was pipetted on to H225 cells. Total luciferase activity of the cells is shown and represents the mean value of the duplicates (±s.d.).

enhancement obtained by the DNA complexation step performed in $NaHCO_3/Na_2HPO_4$.

We have recently shown that fusigenic peptides derived from the N-terminus of hemagglutinin from the influenza virus are able to increase the efficiency of Transfectam/DNA complexes up to 1000 times, when Transfectam is used in suboptimal amounts.[13] Preparing these ternary complexes in $NaHCO_3/Na_2HPO_4$ further improved their efficiency by a factor of seven (Figure 3).

When the cationic lipid was diluted in $NaHCO_3/Na_2HPO_4$ and the DNA in 150 mM NaCl for complexation, the complexes remained very efficient in gene transfer. However, when the lipid was diluted in 150 mM NaCl and the DNA in $NaHCO_3/Na_2HPO_4$, the resulting complexes lost their gene transfer efficacy (Figure 1b). These formulations were then used in a DNA retardation assay (see Figure 4). The following observations could be made: (1) Transfectam 1 eq complexed to DNA in a 150 mM NaCl solution was able to retard all the DNA. The same result was obtained when, before mixing, Transfectam was diluted in NaCl and the plasmid in $NaHCO_3/Na_2HPO_4$; (2) however, with the complexes obtained when the lipid is diluted in $NaHCO_3/Na_2HPO_4$ and the DNA in NaCl or $NaHCO_3/Na_2HPO_4$, only a small part of the DNA is retarded. A similar pattern was observed when 4 charge equivalents of Transfectam were used (Figure 4). Note that a sodium chloride dispersion containing 4 charge equivalents of Transfectam was able to prevent intercalation of ethidium bromide in DNA (Figure 4, lane 2 and 5, lower panel). These results show that there is, depending on the complexation buffer used, an even or uneven distribution of the cationic lipid among the DNA molecules. Thus, a possible explanation for efficient transfection by $NaHCO_3/Na_2HPO_4$ lipoplexes formed at low ratios is that only a small portion of the total DNA is complexed with the cationic lipid to form transfection-competent particles.

Since high reporter gene expression was obtained with Transfectam 0.75 eq/DNA complexes formulated in $NaHCO_3/Na_2HPO_4$ while only a small part of the DNA is complexed, we asked what would happen if the amount of DNA used for transfection were reduced. Figure 5 shows that using 0.5 or 1 µg of DNA per well instead of 2 µg reduces the efficiency of Transfectam 4 eq/DNA (NaCl) and Transfectam 0.75 eq/DNA ($NaHCO_3/Na_2HPO_4$ or NaCl) complexes. However, reporter gene expression obtained with the Transfectam 4 eq ($NaHCO_3/Na_2HPO_4$) complexes remained high.

In the presence of 20% serum (of both heat-inactivated serum and non-decomplemented serum) the transfection efficiency of the $NaHCO_3/Na_2HPO_4$ formulation was affected in a comparable manner to the NaCl preparation (data not shown). Finally, we asked whether the addition of the anionic ions to the lipoplexes has an influence on the size of the DNA complexes. The particle size measurements obtained by laser light scattering showed that the complexes formulated in sodium chloride were smaller than those prepared in $NaHCO_3/Na_2HPO_4$ (400–750 nm *versus* 950–1050 nm).

Taken together, our data show that only a small part of the Transfectam/DNA complexes prepared in 150 mM sodium chloride is involved in gene transfer. Formulation of complexes in $NaHCO_3/Na_2HPO_4$ leads to the same expression levels as Transfectam 4 eq/DNA/NaCl complexes but either less lipid needs to be used or fewer complexes needs to be applied to the cells (see Figure 5). Because there is a dose-dependent toxicity associated with the use of lipoplexes[22,23] (and AK unpublished results), it is advantageous to minimize the amount of complexes and lipid used. In addition, with complexes prepared in sodium chl ride, there is a narrow window

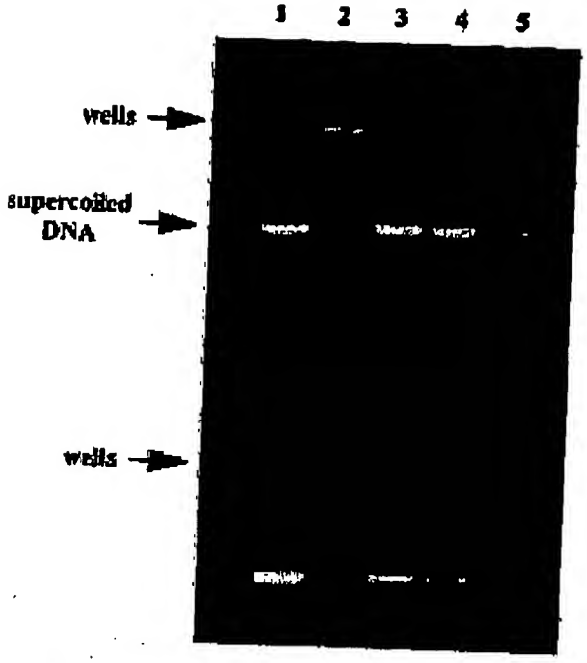

Figure 4 Analysis of the interaction between plasmid DNA and Transfectam by gel retardation assay. One microgram plasmid DNA in 25 µl buffer (150 mM NaCl or 23.8 mM $NaHCO_3$/5.6 mM Na_2HPO_4) was mixed with aliquots of lipid (1 or 4 charge equivalents of Transfectam) also diluted in 25 µl of buffer. After a 10-min maturation period, 25 µl of the complexes were then applied on an agarose gel. The gel was prepared with 0.9% agarose in Tris-EDTA with 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 70 V and 35 mA for 80 min. Lane 1, plasmid; lane 2, Transfectam/NaCl + DNA/NaCl; lane 3, Transfectam/$NaHCO_3$-Na_2HPO_4 + DNA/$NaHCO_3$-Na_2HPO_4; lane 4, Transfectam/$NaHCO_3$-Na_2HPO_4 + DNA/NaCl; lane 5, Transfectam/NaCl + DNA/$NaHCO_3$-Na_2HPO_4. In the upper panel 1 eq of Transfectam was used; for the lower panel Transfectam was used at 4 charge equivalents.

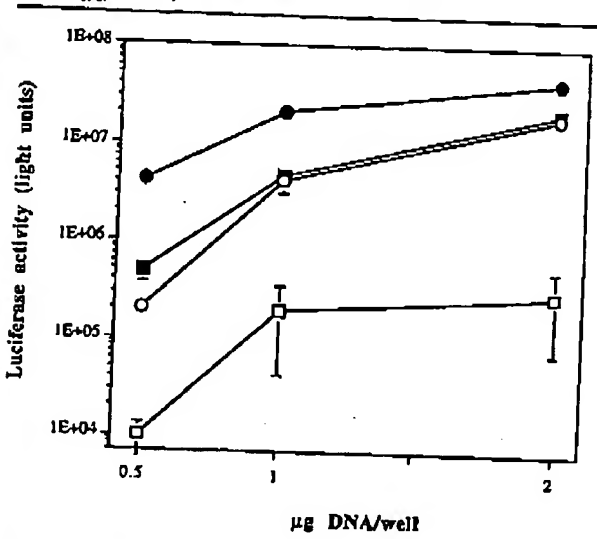

Figure 5 Transfection levels obtained with decreasing amounts of plasmid DNA depend on the condensation buffer. HepG2 cells were transfected with decreasing amounts of lipoplexes. The complexes of 0.75 (-□-), and 4 (-○-) charge equivalents were prepared either in 0.15 M sodium chloride (open symbols) or in $NaHCO_3$/Na_2HPO_4 (closed symbols). Total luciferase activities per well are shown and are the means of the duplicates (±s.d.).

for efficient transfection – ie conditions outside those optimal, result in a considerable decrease of reporter gene expression. In contrast, the window is much larger with transfection particles prepared in RPMI medium or $NaHCO_3$/Na_2HPO_4. This point could be particularly important for *in vivo* applications. The isolation and characterization of these highly efficient Transfectam/DNA/$NaHCO_3$-Na_2HPO_4 particles will be the focus of future investigations.

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